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The current work has the overall objectives of genetically engineering mono- and poly-valent, IL-12 producing vaccinia/*Brucella* recombinant vaccines using synthetic E/L promoters and to test such vaccinia recombinants for their ability to induce a protective immune response against challenge with virulent *Brucella* in a mouse model. For an effective and strongly protective immune response it may be necessary to combine vaccination with vaccinia/*Brucella* recombinants with the inoculation of DNA vaccines. At this stage the following has been achieved: 1. Demonstrated *Brucella* Cu/Zn SOD to be a protective antigen. 2. Produced first WRvaccinia/*Brucella* antigen recombinants simultaneously producing IL-12. 3. Produced first WRvaccinia/*Brucella* recombinants producing a fusion protein of two protective *Brucella* antigens (SOD and L7/L12). 4. Initiated protection studies with WRvaccinia/*Brucella* antigen recombinants producing IL-12. 5. Detected additional protective *Brucella* antigens (BMCP24, BaBSCP31 and BaPAL16.5) ready for cloning into vaccinia. 6. Produced several *Brucella* DNA vaccines to be used in combination with WRvaccinia/*Brucella* antigen recombinants and 7. Initiated production of MVA vaccinia/*Brucella* antigen recombinants. Based on the identification of protective antigens and the role of IL-12, we are confident that the use of vaccinia/*Brucella* recombinants producing IL-12 will induce protective immunity. We are now able to produce such recombinants.

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4. TABLE OF CONTENTS.

5. INTRODUCTION.

The current work has the overall objectives of genetically engineering mono- and poly-valent, IL-12 producing vaccinia/Brucella recombinant vaccines using synthetic E/L promoters and to test such vaccinia recombinants for their ability to induce a protective immune response against challenge with virulent Brucella in a mouse model. If such vaccines are able to protect mice against challenge with Brucella there is a good possibility that such vaccines could be used to immunize humans against infection with Brucella spp. For an effective and strongly protective immune response it may be necessary to combine vaccination with vaccinia/Brucella recombinants with the inoculation of DNA vaccines.

6. BODY.

Approved objectives of the project:

a. Construct vaccinia virus/Brucella recombinants expressing one or more demonstrated protective Brucella antigens (SOD & L7/L12 proteins) and potentially protective antigens (14 kDa protein, 18 kDa lipoprotein, YajC and/or newly detected antigens) . Use vaccinia strains Western Reserve (WR - with and without expression of IL-12) and modified vaccinia virus Ankara (MVA) strain.

b. Construct similar vaccinia virus/Brucella recombinants expressing combinations of the above Brucella antigens simultaneously.

c. Immunize BALB/c mice with the vaccinia/Brucella recombinants and analyze the humoral and CMI responses to Brucella antigens. Challenge vaccinated mice with virulent Brucella spp. and test the ability of the vaccinations to induce protection.

This project was initiated in June 1998. It was intended that the initiation of this project would have occurred immediately or shortly after the first funded project was completed in order to maintain the aquiered scientific momentum and maintain the same research staff. Unfortunately this did not occur creating a significant interruption of the ongoing work with reassignment of the personell to other projects. Once funding was approved, a new effort had to be put forward to hire new staff and restart the work.

Under the previous project, vaccinia constructs were made using a variety of Brucella genes. Immunization of mice with these constructs did not protect the animals against challenge with a virulent Brucella strain.

One possible reason for failure was that non-protective Brucella antigens had been cloned into the vaccinia constructs. We have now demonstrated that at least one of the antigens used, Brucella Cu/ZN SOD, is a protective antigen if applied as a recombinant antigen using E. coli as a vector (1) or as a purified recombinant protein (2). Therefore, we now have 2 antigens, Brucella Cu/ZN SOD and L7/L12 with proven protective abilities (1,3). Appropriate vaccinia recombinants expressing either or both of these antigens should therefore be protective vaccines if antigens are presented appropriately to the immune system. Previous results from our studies indicated that vaccinia WR expressing Brucella SOD under the control of a variety of promoters, did not protect mice from challenge. The combined findings strongly suggests that the vaccinia vectors selected in the previous studies did not have the ability to induce the correct, protective cell mediated immune responses.

Purified, recombinant SOD is able to induce a mild protective response to Brucella infection in mice (2). This protective response is significantly increased if SOD is administered with IL-12 (2). Recombinant L7/L12 ribosomal protein is not able to induce a protective response in mice if injected as a purified protein alone but, if injected with IL-12, it induces a significant protective response (2). These recent findings lend support to our assumption that WR vaccinia recombinants can not induce a protective anti-Brucella response even if they express the correct protective antigen(s) unless IL-12 is simultaneously produced to induce an effective and protective CMI response. Therefore, we have now concentrated our work in producing such recombinants. Recent information regarding vaccinia/HIV-1 Env recombinants indicate that simultaneous production of IL-12 by the recombinant or mixing of the recombinant vaccinia with a vaccinia producing IL-12 (co-administration) will induce the highest specific CMI (4).

Recent information also indicates that DNA vaccines combined with vaccinia recombinants can induce very strong protective immunity even if each by itself does not or induces very weak immunity (5). Therefore, we have also produced DNA vaccines with selected Brucella antigens of potential protective abilities to

a) increase our pool of known protective antigens and
b) combine such vaccines with our vaccinia recombinants.

We are demonstrating that combination of Brucella DNA vaccines with other Brucella vaccines also enhances anti-Brucella immunity since selected Brucella DNA vaccines with very marginal protective abilities by themselves can significantly increase protection of B. abortus vaccine strain RB51. These findings suggest that the combination of IL-12 producing vaccinia/Brucella recombinants with DNA vaccines will enhance protective immunity. Experiments along this line are therefore warranted.

Constructs.

1. Vaccinia/Brucella recombinants.

a. pMCO2/SOD vaccinia shuttle vector:

A 600-bp fragment containing the B. abortus sodC gene (solid arrow) was cloned into the shuttle vector pMCO2 (figure 1).

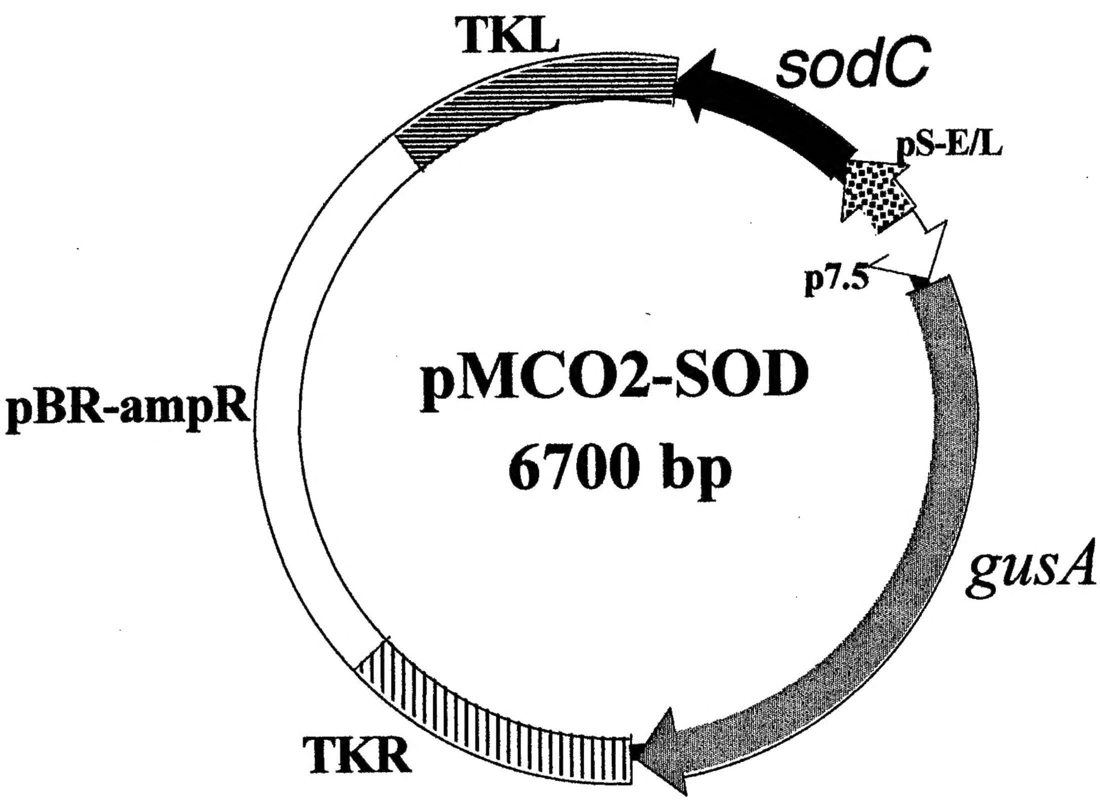

Figure 1. Schematic diagram of shuttle vector used to construct recombinant vaccinia virus expressing Brucella Cu/Zn SOD. Similar shuttle vectors are used to construct remaining vaccinia/ Brucella antigen recombinants.

The synthetic early/late vaccinia virus promoter pS-E/L (dotted arrow) drives expression of the sodC gene and a natural early/late vaccinia viral promoter p7.5 (open arrow) drives the expression of glucoronidase enzyme (gray arrow). The vaccinia virus thymidine kinase sequences (TKL and TKR) flank the expression cassette. Sequences required for the replication in E. coli and resistance to ampicillin are indicated (open bar). WR/pMC02-SOD was

constructed and is being used in combination with a DNA-Brucella SOD vaccine. The following experiment is in progress and results will be available on 8/9/99.

Table 1. Mouse protection/challenge experiment with Vaccinia/Brucella-SOD recombinant in combination with DNA-SOD vaccine (ongoing).

Group #	# of mice	Vaccine and schedule
1	5	WR/pMC02-SOD day0 & DNA Vaccine week 7
2	5	DNA Vaccine day0 & WR/pMC02-SOD week 7
3	5	RB51 (positive control) week 7
4	5	Saline (negative control)

Challenge week 14 and termination week 16.
See DNA vaccines for details of construction for DNA-SOD vaccine.

The results of this experiment will tell us if the combination of a vaccinia recombinant expressing a protective Brucella antigen and which has not been able to induce protection by itself, can lead to protection if combined with a DNA vaccine of the same antigen (which by itself alone does not produce protective immunity either).

b. Vaccinia/Brucella recombinants producing IL-12.

The following have been constructed and mice protection experiments have been initiated or are being initiated. One problem, which has lead to several delays with these constructs, has been that the recombinants are often lost during tertiary enhancement steps. This often forces us to repeat the selection process; we have yet to define the exact reason(s) leading to problems with the enhancement steps.

WRSOD::L7/L12 (WR vaccinia expressing SOD and L7/L12 as a fusion protein)
WRSOD::L7/L12;IL-12 (WR vaccinia expressing SOD and L7/L12 as a fusion protein and expressing functional IL-12).
WRSOD/IL-12 (WR vaccinia expressing SOD and functional IL-12)
WRLacZ/IL-12 (WR vaccinia expressing LacZ and functional IL-12).
Under construction are:
WR SOD/L7/L12 (WR expressing SOD and L7/L12 as separate proteins) and
WRSOD/L7/L12/IL-12 (WR expressing SOD, L7/L12and IL-12 as separate proteins).

At present, a pilot protection experiment with some of these constructs (WRSOD::L7/L12; WRSOD::L7/L12;IL-12; WRLacZ/IL-12 and appropriate

controls has been initiated in mice).

The following vaccinia virus strains were used or will be used to generate recombinants: vaccinia virus strain WR, vaccinia virus strain WR expressing IL-12 (vMCB7.1/IL-12) and vaccinia virus strain MVA. The highly attenuated MVA strain will be used for the expression of Brucella antigens which demonstrated protective and/or CMI inducing abilities in mice vaccinated with any of the WR vaccinia virus recombinants expressing the corresponding Brucella antigen.

Work has been initiated to prepare MVA recombinants. Chicken fibroblast cell cultures necessary for the propagation of these recombinants have been started.

Cloning procedures that have been well standardized in our laboratory have been followed to create the recombinant vaccinia viruses in Hutk- and CV-1 cells as described for the WR strain (6) and chicken embryo fibroblasts for the MVA strain (7). Recombinant transfer vectors containing the Brucella genes will be used to transfect cells infected with vaccinia virus (strain WR, vMCB7.1/IL-12 or MVA). The vMCB7.1/IL-12 virus (obtained from M. Carroll, NIH) contains the two subunit genes of murine IL-12 in its HA (hemagglutinin) locus and expresses functional IL-12. Recombinant viruses will be isolated by three rounds of plaquing and enhancement (6) and will be checked for the expression of respective Brucella genes by performing Western blotting with specific anti-Brucella sera available in our center. Either pMCO2, pSC65 or pSC11 plasmid transfer vectors will be used for creating the recombinant vaccinia WR strain viruses. Since insertion of a foreign gene in the tk locus of MVA strain of vaccinia virus results in poor production of recombinants (8) the plasmid vector pMCO3 (9) will be used for this purpose. This transfer vector allows the insertion of foreign genes into the deletion III site of the MVA strain genome. Like with pMCO2 and pSC65, expression of foreign gene in pMCO3 is also driven by a strong E/L synthetic promoter.

In general, the following approaches have been and are being used to characterize the immune response and to detect protection in mice.

To characterize the humoral and CMI responses induced by vaccinia virus/Brucella recombinants and DNA vaccines, BALB/c mice are inoculated. Serum is obtained for western blot analysis. CMI responses are determined with spleen lymphocytes using in vitro assays able to detect: lymphocyte proliferation (LTA) (10), cytokine production (particularly INF-g) by ELISAs (11) and specific cytotoxic T cell activity using 51Cr or neutral red labeled Brucella infected syngeneic macrophage cell lines as targets (12). The tests are carried out using whole Brucella and Brucella fractions as antigens as well as specific, purified recombinant antigen preparations obtained as pMAL fusion proteins. These tests will indicate if antibodies and/or Brucella specific

sensitized T cells have been induced by the recombinant vaccine.

Protection experiments are carried out as described (13). Briefly, mice will be immunized with selected recombinant vaccines and appropriate controls. The mice are challenged with virulent B. abortus strain 2308 or B. melitensis strain 16M 6 to 7 weeks post-immunization. Mice are killed two weeks post challenge and splenocytes are cultured to determine the cfu of Brucella/ spleen. Comparing cfu of vaccinated animals with controls using a Student's t test will assess protection .

c. DNA vaccines.

Initially, based on the published literature, we selected 10 Brucella proteins which showed the potential to induce an immune response (table 2). We retrieved the gene sequences of the 10 proteins from the databases. For each gene, we designed a pair of primers to PCR amplify the complete open reading frame. A restriction enzyme site has been engineered into each of the primers to facilitate directional cloning into pCDNA3, a mammalian expression vector. Selection of the restriction enzyme sites is based on the availability of such sites in the multiple cloning site of pCDNA3 and at the same time, allows us to subclone in the vaccinia shuttle vectors. The genes were PCR amplified using the designed primers and genomic DNA of either B. abortus or B. melitensis as template. Of the ten candidate antigens, six are from B. abortus and the other four are from B. melitensis. PCR products were purified and cloned into pCR2.1 (TA Cloning, Invitrogen). Each of the selected genes was subsequently subcloned into pcDNA.. Each of the DNA vaccine constructs were then prepared by large-scale purification using Qiagen Endo Free kits. Injections are given IM and/or ID. Additionally we constructed DNA vaccine for SOD and L7/L12. These will be used in protection/challenge experiments with vaccinia/ Brucella SOD and L7/L12 recombinants.

The 10 DNA vaccines prepared were tested in mice for their ability to induce protection alone or in combination with vaccine RB51(table 2). When combining with RB51 we were looking for enhancement of protection as compared to RB51 alone. The 3 DNA vaccines which gave some marginal protection (but significant – we are repeating experiments with these ones) enhanced RB51 protection very strongly. Some DNA vaccines, which by themselves did not induce protection, did enhance the protective effects of RB51 somewhat. At present we are concentrating on BMCP24, BaBSCP31 and BaPAL16.5 for use in vaccinia recombinants (in addition to SOD and L&/L12) particularly in recombinants expressing multiple Brucella antigens.

Table 2. Genes selected for DNA vaccines in addition to Brucella Cu/Zn SOD and L7/L12 .

Gene	Protein/antigen coded for	Protection as DNA vaccine Alone	Enhances RB51 protection
1. Bm CP24	ribosome releasing factor	YES	YES*
2. Ba BSCP31	surface protein	YES	YES*
3. Ba PAL16.5	lipoprotein	YES	YES*
4. Ba catalase	catalase enzyme	NO	NO
5. Bm bfr	bacterioferritin	NO	NO
6. Ba 26 kDa	periplasmic protein	NO	YES
7. Ba 25 kDa	immunogenic in sheep	NO	YES
8. Ba 10 kDa	outer membrane lipoprotein	NO	YES
9. Bm 31 kDa	protein (major omp)	NO	YES
10. Bm 28 kDa	cytoplasmic protein	NO	NO
11. pcDNA3	(vector control)	NO	NO

*= very significant enhancement of protection.
Bm: denotes gene from B. melitensis
Ba: denotes gene from B. abortus

7. KEY RESEARCH ACCOMPLISHMENTS DURING THIS PROJECT.

- Demonstrated Brucella Cu/Zn SOD to be a protective antigen.
- Produced first WRvaccinia/Brucella antigen recombinants simultaneously producing IL-12.
- Produced first WRvaccinia/Brucella recombinants producing a fusion protein of two protective Brucella antigens (SOD and L7/L12).
- Initiated protection studies with WRvaccinia/Brucella antigen recombinants producing IL-12.
- Detected additional protective Brucella antigens (BMCP24, BaBSCP31 and BaPAL16.5) ready for cloning into vaccinia.
- Produced several Brucella DNA vaccines to be used in combination with WRvaccinia/Brucella antigen recombinants.
- Initiated production of MVA vaccinia/Brucella antigen recombinants.

8. REPORTABLE OUTCOMES.

All manuscripts published and abstracts presented on WRvaccinia/ Brucella antigen recombinants were produced under the previous grant. At present this project is supporting (with supplies only –stipend provided by the College) one Ph.D. thesis on WRvaccinia/ Brucella antigen recombinants. Part of the approach was presented at the Brucellosis Research Conference in Chicago in November 1988 : S. Baloglu, R. Vemulapalli, S. M. Boyle, T. Toth,

N. Sriranganathan, T. Toth, and G. Schurig. Vaccinia virus as a vector for delivery of Brucella antigens: Recent observations. S. Baloglu, R. Vemulapalli, S. M. Boyle, N.
We expect to present the newly obtained data this November at the Brucellosis research Conference in Chicago.

9. CONCLUSIONS.

Previous and present work indicates that WRvaccinia/ Brucella antigen recombinants are not conferring protective immunity . This is not necessarily due to promoter selection as demonstrated in the previous project. Based on the finding that Brucella Cu/ZN SOD and L7/L12 protein are protective antigens and that presence of IL-12 is necessary at the moment of antigen presentation, we are confident that the use of vaccinia/Brucella recombinants producing IL-12 will induce protective immunity. We are now able to produce such recombinants. Expression of more than one protective antigen in addition to IL-12 should further increase protective immunity and combination with the appropriate DNA vaccines may lead to sterile immunity. It should be kept in mind that simultaneous expression of multiple protective Brucella antigens may be a requirement for achieving high protective levels in all individuals of a population.

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